Quantitative analysis of exocytosis visualized by a video-enhanced light/fluorescence microscope reveals two distinct components of exocytosis in RBL-2H3 cells

Koichiro Ozawa[a,*], Hideyuki Kobayashi[a], Eriko Kawai[a], Etsuko Suzaki[a], Yoshiaki Nonomura[b], Tsutomu Masujima[a]

[a]*Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan*
[b]*First Department of Pharmacology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan*

Received 5 August 1996; revised version received 8 October 1996

Abstract Rat basophilic leukemia (RBL-2H3) cells, which exhibit Ca^{2+}-dependent secretion of granules when stimulated with antigen or the Ca^{2+}-ionophore A23187, were observed under a video-enhanced light/fluorescence microscope. Exocytotic events of individual granules were visualized in individual cells stimulated with antigen or A23187. Exocytosis of granules stimulated with A23187 showed two peaks in the time course. The earlier one was inhibited by selective inhibitors of protein kinase C (Ro31-8425, Ro31-8220, and chelerythrine) and the other was inhibited by an inhibitor of phosphatidate hydrolase, propranolol. Exocytosis by antigen stimulation, however, showed only one peak, which was inhibited by the selective inhibitors of protein kinase C, but not by propranolol. These results indicate that at least two distinct components of exocytosis exist in RBL-2H3 cells.

Key words: Exocytosis; Video-microscope; Protein kinase C; Phospholipase D; RBL-2H3 cell

1. Introduction

Secretory events of granules, which contain hormones and transmitters, in response to an appropriate stimulus are thought to be mediated by exocytosis, a fusion of secretory granules with the plasma membrane, an extrusion of the contents to the extracellular space, and a membrane retrieval. Recent studies with video-microscopic techniques employing a charge coupled device (CCD) camera and/or a silicon intensified target (SIT) camera, an image processor, and a videotape recorder have determined the properties and mechanisms of exocytosis in many cells, including adrenal chromaffin cells [1] mast cells [2], colonic goblet cells [3], and salivary glands [4].

Rat basophilic leukemia (RBL-2H3) cells, a tumor analog of rat mucosal mast cells [5], have been widely used as a model for the study of mechanisms underlying the exocytotic process [6]. These cells can be stimulated to secrete their intracellular granules in response to the cross-linking of ($Fc_{\varepsilon}RI$ category) receptor-bound immunoglobulin E (IgE) with antigen, initiating a cascade of events [7] which include the activation of tyrosine kinases, Lyn and Syk [8], and the tyrosine phosphorylation of various proteins [9–11], including phospholipase Cγ1 [12] and phosphatidylinositol 3-kinase (PI3-kinase) [13], the activation of phospholipase C [12] and phospholipase D (PLD) [14,15], along with sustained elevation of diglycerides [15] and mobilization of Ca^{2+} from intracellular and extracellular sources [6,16,17], followed by the activation of several serine/threonine kinases including protein kinase C (PKC) [18,19], Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) [20,21], and a mitogen activated protein kinase [22,23].

Recently, the application of patch clamp techniques to monitor the activity of individual fusion pores in mast cells has generated a wealth of novel and unexpected observations underlying exocytosis in many cells, including mast cells, neutrophils, adrenal chromaffin cells, and pituitary cells (see [24,25] for reviews). Patch clamp measurements of secretory cells undergoing exocytosis have revealed the exocytotic fusion pore as a remarkably dynamic entity. With this technique, it has been clarified that the molecular structures that regulate membrane fusion are likely to contain related proteins, i.e. Rab3 proteins, and fundamental properties. The patch clamp techniques have been used for quantitative analysis of exocytosis from granules, which contain hormones and transmitters, in response to an appropriate stimulus in many cells, including mast cells [24,25], but it has not been reported in RBL-2H3 cells. For the quantitative analysis of exocytosis in RBL-2H3 cells, measurements of biogenic amines (i.e. histamine and 5-hydroxytryptamine) or hexosaminidase release from the cells have been widely used [7–23]. Recent advances in measuring low concentrations of biogenic amines using voltammetric techniques have allowed measurement of 5-hydroxytryptamine from single mast cell granules [26], but a single cell technique for measurement of secretion from RBL-2H3 cells is not available.

Here we report the visualization of contents release from low-pH granules, including histamine and 5-hydroxytryptamine, to the extracellular space in RBL-2H3 cells by a video-enhanced light/fluorescence microscope. We also demonstrate, with quantitative analysis of single cell exocytosis directly visualized by the microscope, that PKC and PLD were involved in antigen- and A23187-induced exocytosis not only through distinct pathways but also with different time courses. It was also suggested that the visualization of secretory processes by the video-enhanced light/fluorescence

*Corresponding author. Fax: (81) (82) 257-5304.

Abbreviations: RBL-2H3 cells, rat basophilic leukemia 2H3 cells; IgE, immunoglobulin E; $Fc_{\varepsilon}RI$, high affinity immunoglobulin E receptor; DNP, 2,4-dinitrophenol; DNP-BSA, antigen consisting of 24 molecules of dinitrophenol conjugated with one molecule of bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid; PI3-kinase, phosphatidylinositol 3-kinase; MLCK, myosin light chain kinase; PKC, protein kinase C; PLD, phospholipase D; CCD camera, charge coupled device camera; SIT camera, silicon intensified target camera; DIC, differential interference contrast

PII S0014-5793(96)01184-2

microscope might be useful for the study of molecular mechanisms underlying exocytosis and other cellular events.

2. Materials and methods

Materials were obtained from the following sources: DL-propranolol and calcium ionophore A23187 from Sigma, St. Louis, MI; calphostin C from Biomol Research, Plymouth Meeting, PA; Ro31-8220 and chelerythrine chloride from Calbiochem-Novabiochem Int., San Diego, CA. The antigen, DNP-BSA, and DNP-specific monoclonal IgE were kindly supplied by Dr. Henry Metzger (NIAMS, National Institutes of Health). Ro31-8425 was a gift from Eisai Co., Ibaraki. Other reagents and materials were from the sources listed previously [18].

Stock solution of Ro31-8425, Ro31-8220, chelerythrine, calphostin C, and A23187 were prepared in dimethyl sulfoxide and diluted to give $<0.1\%$ dimethyl sulfoxide [27]. All other reagents were dissolved in a PIPES-buffered saline (see below).

2.1. Preparation of cell cultures for experiments

For observation under a video-enhanced light/fluorescence microscope, RBL-2H3 cells were incubated overnight with 0.5 μg/ml of DNP-specific IgE on coverslips (8×10^4 cells/ml of medium/slip). Cultures were washed and experiments were performed with a PIPES-buffered saline (25 mM PIPES, pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 5.6 mM glucose, 1 mM $CaCl_2$ and 0.1% BSA), as required, 0.1 mM EGTA was added instead of Ca^{2+} (Ca^{2+}-free medium).

2.2. Observation with a video-enhanced light/fluorescence microscope

Observation with a video-enhanced light/fluorescence microscope was performed with minor modifications of previously described techniques [28]. Specifically, RBL-2H3 cells on a coverslip were washed with a PIPES-buffered saline and left for 10 min on a tissue culture dish containing the medium. The coverslip was mounted on a perfusion chamber and continuously perfused with the medium. Stimulation of the cells with antigen or A23187 was performed by perfusion. Differential interference contrast (DIC) images and fluorescence images were observed with a Zeiss axiovert 135 TV microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CCD camera (CS8330; Tokyo Electronic Industry, Tokyo) for the DIC image and/or a SIT camera (C2741; Hamamatsu Photonics, Hamamatsu) for the fluorescence image, an image processor (PIP-4000; A.D.S., Tokyo), and a video tape recorder (AG-7355; Panasonic, Osaka). The video signal of the camera or the recorded data was contrast-enhanced with the image processor.

For observation of the secretory process using a fluorescent aminoacridine dye, quinacrine, which was reported to be incorporated selectively into low-pH secretory granules in RBL-2H3 cells and whose incorporation has been used for quantitation of exocytosis in this cell line [29], a band pass filter (peak transmission wavelength 430 nm) and a long pass filter (half-transmission wavelength 470 nm) were used for the excitation side and the emission side, respectively, while motion of granules was observed through the DIC image.

All measurements were performed at room temperature (25–26°C).

2.3. Staining with quinacrine

As required, RBL-2H3 cells on coverslips were stained with 0.5 μM of quinacrine for 5 min at 37°C in the PIPES-buffered saline. Then, they were washed with the medium and perfused immediately. Quinacrine itself, even at 5 μM, did not affect exocytosis (data not shown).

2.4. Scoring number of exocytosis through the DIC image

Determination of the rate of exocytosis in RBL-2H3 cells through the DIC image recorded in the videotape was performed with previously described methods [28]. Specifically, the number of granules (total granules), which were 0.5-0.8 μm in diameter and pre-stained with quinacrine, in a fixed focal plane of a cell (around 1 μm deep from the cell surface) was counted just before the beginning of exocytotic events. The mean value of the total number of granules, not in the whole cell but in a fixed focal plane, in each experiment was 74 (60–101). Then, the number of granules on which the exocytotic events were observed was also counted (exocytotic granules). The rate of exocytosis was expressed as the ratio of the number of exocytotic granules to that of total granules. Although we measured the rate of exocytosis not in the whole cell but in a fixed focal plane, the ratio of exocytosis calculated with this method was similar, but somewhat low, to the results from β-hexosaminidase or histamine release (data not shown, but similar results were previously reported in [18,27]). The exocytotic response of RBL-2H3 cells was not an 'all-or-none' type of response, but some cells did not show exocytosis even after the proper stimulation through all experiments; the rate of non-responding cells was 8.8% (10 of 114 cells) and 6.9% (8 of 116 cells) for antigen or A23187 stimulation, respectively. In all results of quantitative analysis, the data of non-responding cells were omitted from the results.

3. Results

3.1. Visualization of exocytotic process in RBL-2H3 cells

When RBL-2H3 cells were observed under a Nomarski microscope through a CCD camera, an image processor, and a videotape recorder, exocytotic events of individual granules were visualized in individual cells stimulated with 20 ng/ml of DNP-BSA (Fig. 1a). Initial steps were quicker than 33 ms (Fig. 1b). The exocytotic events stimulated with antigen were not observed in Ca^{2+}-free medium (data not shown). To discover whether the release of contents of a single granule can be visualized, we tried with a fluorescent aminoacridine dye, quinacrine, which is known to be incorporated selectively into low-pH secretory granules, including histamine, 5-hydroxytryptamine, and β-hexosaminidase, in RBL-2H3 cells [29]. The granules stained with quinacrine emitted bluish green fluorescence. As shown in Fig. 2, upon stimulation with antigen, release of the quinacrine fluorescence from a granule was observed, while exocytotic events, shown through the DIC image, were observed on the same granule (Fig. 2).

In another series of experiments, antigen (Fig. 3a,b) or A23187 (Fig. 3c,d) showed a time- and concentration-dependent exocytosis. It is noteworthy, as shown in Fig. 3a,c, that exocytosis of granules stimulated with A23187 showed two

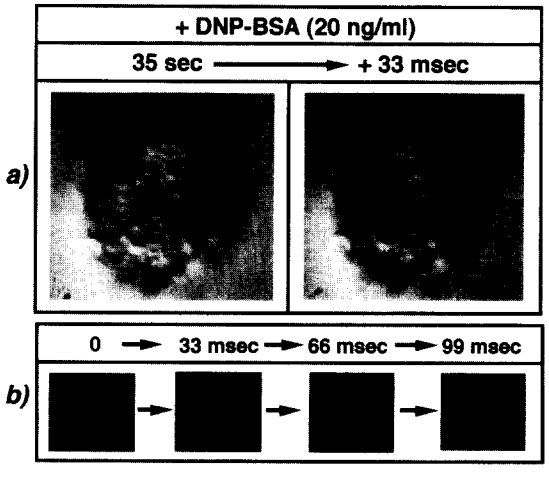

Fig. 1. Nomarski images of exocytosis induced by antigen. RBL-2H3 cells on coverslips were washed and perfused with PIPES-buffered saline, and then the cells were observed with a video-enhanced light/fluorescence microscope. RBL-2H3 cells were incubated with 20 ng/ml of DNP-BSA for the indicated times. a: Images of a cell reproduced from video frames with a 33 ms interval. Granules (arrows) showed abrupt increases in light intensity. b: Difference image obtained by subtracting the image of one frame from the previous frame. The interval between frames is 33 ms. This clearly reveals the rapid brightness change of a granule (arrows in a).

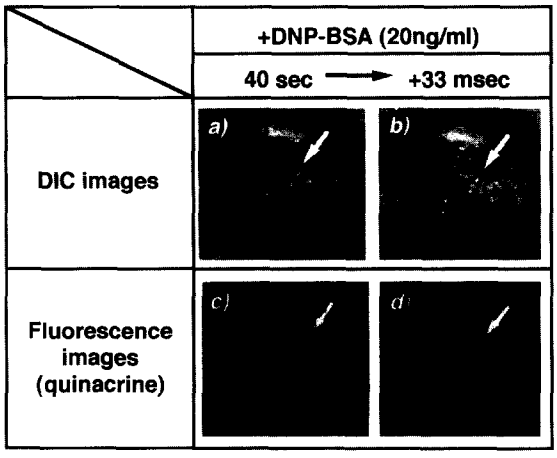

Fig. 2. Release of quinacrine accumulated in low-pH granules and Nomarski images of exocytosis in RBL-2H3 cells. RBL-2H3 cells on coverslips were stained with 0.5 μM of quinacrine for 5 min at 37°C in PIPES-buffered saline and washed by perfusion, and then the cells were observed with a video-enhanced light/fluorescence microscope. The cells were stimulated with 20 ng/ml of DNP-BSA. a and b: Images of a cell reproduced from video frames with a 33 ms interval. A granule (arrow) showed abrupt increases in light intensity. c and d: Fluorescence at 500 nm was measured. Release of quinacrine fluorescence from a granule (arrow) was observed in the same granule, shown in a and b, at the same time.

peaks in its time course but antigen-induced exocytosis showed only one. With increasing the concentrations of antigen or A23187, the maximum points of peak I (Fig. 3a) or peak II (Fig. 3c) were shifted from 1.5 min to 1 min and from 1 min to 30 s, respectively. Maximal responses of exocytosis with antigen or A23187 were 63% of the total and 42%, respectively. These results are similar to the results of release of histamine, 5-hydroxytryptamine, or β-hexosaminidase in previous reports [13,18,19,28,30].

3.2. Evidence that PKC and PLD were involved in exocytosis

To determine the meaning of the difference between antigen- and A23187-induced exocytosis in its time course, we studied the effects of inhibitors of PKC as well as phosphatidate hydrolase on the exocytosis stimulated with DNP-BSA or A23187. RBL-2H3 cells, sensitized with DNP-BSA-specific IgE, on a coverslip were washed and incubated with various concentrations of inhibitors at 37°C for 10 min, and the coverslip was mounted on a perfusion chamber and continuously perfused with the medium containing the same concentrations of the inhibitors. The cells were immediately stimulated with DNP-BSA or A23187. Ro31-8425, a selective inhibitor of PKC, inhibited antigen-induced exocytosis (Fig. 4a); the IC_{50} value was calculated to be 0.2 μM (Fig. 4b). Ro31-8220 and chelerythrine, selective inhibitors of PKC, also inhibited antigen-induced exocytosis at a concentration of 10 μM (Fig. 5a,b); the IC_{50} values were calculated to be 0.5 μM and 0.8 μM for Ro31-8220 and chelerythrine, respectively. Although calphostin C, which inhibits diacylglycerol binding not only to PKC [31] but also to at least one other protein that is critical for actin polymerization [32], also inhibited antigen-induced exocytosis (Fig. 6a,b), propranolol, an inhibitor of phosphatidate hydrolase, did not affect antigen-induced exocytosis (Fig. 7a,b). For A23187-induced secretion, the results were more complicated. Exocytosis of granules stimulated with A23187 showed two peaks in its time course (Fig. 3c). The earlier peak was inhibited by Ro31-8425 (Fig. 4c), Ro31-8220 (Fig. 5c), or chelerythrine (Fig. 5c) and the

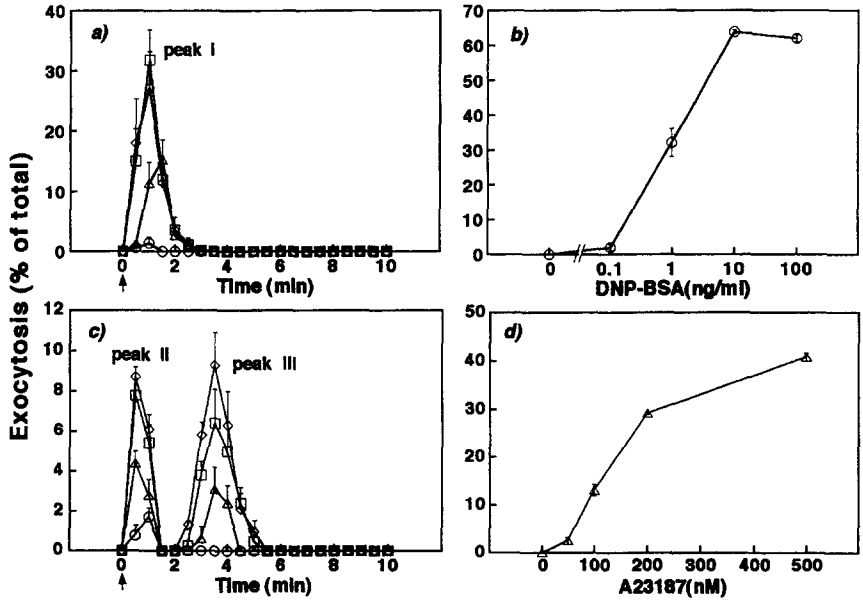

Fig. 3. Effects of antigen and A23187 on exocytosis visualized by a video-enhanced light/fluorescence microscope in RBL-2H3 cells. RBL-2H3 cells on coverslips were washed and perfused with PIPES-buffered saline, and then the cells were observed through a video-enhanced light/fluorescence microscope. RBL-2H3 cells were stimulated with 0.1 (○), 1 (△), 10 (□), or 100 (◇) ng/ml of DNP-BSA in a and with 50 (○), 100 (△), 200 (□), or 500 (◇) nM of A23187 in c. DNP-BSA or A23187 was added at the time indicated by arrows. The ratio of exocytosis (% of total) was calculated by the methods described in Section 2. The mean values of total numbers of granules in each experiment (see Section 2) were 73, 66, 70, or 68 for 0.1, 1, 10, or 100 ng/ml (a) and 66, 67, 75, or 77 for 50, 100, 200, or 500 nM (c), respectively. a and c: Time course of antigen- or A23187-induced exocytosis. b and d: Dose-response curves of antigen- or A23187-induced exocytosis. The ratio of exocytosis in b and d was recalculated as the sum of exocytosis of each dose shown in a and c, respectively. Values were mean ± S.E. (error bars) of seven separate experiments.

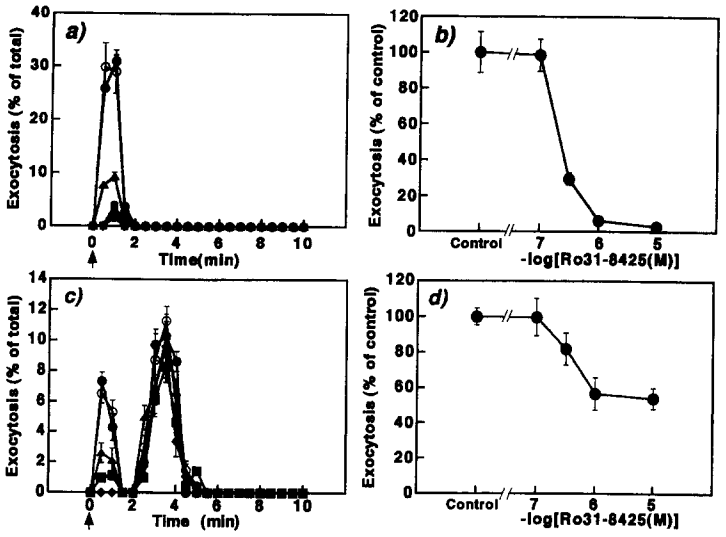

Fig. 4. Effect of Ro31-8425 on antigen- or A23187-induced exocytosis. RBL-2H3 cells on coverslips were pre-incubated with inhibitors for 10 min at 37°C in PIPES-buffered saline and washed by perfusion, and then the cells were observed under a video-enhanced light/fluorescence microscope. The cells were stimulated with 20 ng/ml of DNP-BSA (a) or 500 nM of A23187 (c). DNP-BSA or A23187 was added at the time indicated by arrows. The ratio of exocytosis (% of total) was calculated by the methods described in the legend of Fig. 3. a and c: Time courses of antigen- or A23187-induced exocytosis without (○) or with 0.1 (●), 0.3 (▲), 1 (■), or 10 (◆) μM of Ro31-8425. b and d: Dose-response curves of Ro31-8425 on antigen- or A23187-induced exocytosis. The ratio of exocytosis in b and d was recalculated as the sum of exocytosis of each dose shown in a and c, respectively. Control in b and d means without Ro31-8425 in a and c, respectively. The mean values of total numbers of granules in each experiment were 70–95 (a) and 66–77 (c). The number of granules in Ro31-8425-inhibited cells was not changed before and after the stimulation with antigen or A23187. Values were mean ± S.E. of four separate experiments.

other was inhibited by propranolol (Fig. 7c); IC_{50} values were calculated to be 0.4 μM, 0.6 μM, 0.7 μM, and 0.25 μM for Ro31-8425 (Fig. 4d), Ro31-8220, chelerythrine, and propranolol (Fig. 7d), respectively. Calphostin C, furthermore, inhibited both peaks (Fig. 6c) in a concentration-dependent manner (Fig. 6d). These results indicate that A23187-induced exocytosis is mediated through activation of both PKC (peak II in Fig. 3c) and PLD (peak III in Fig. 3c) and that antigen-induced exocytosis is caused mainly by activation of PKC (peak I in Fig. 3a).

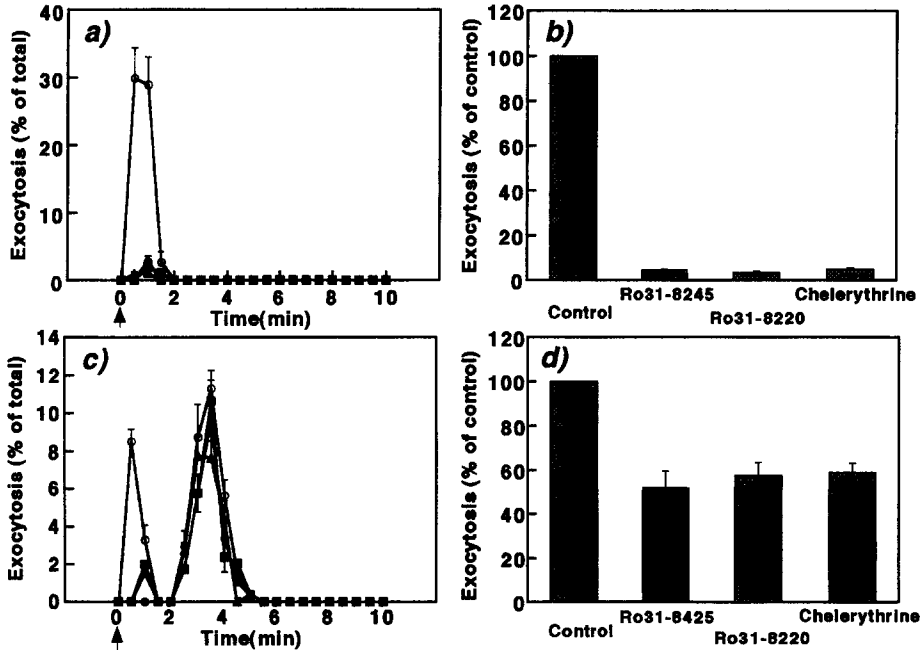

Fig. 5. Effect of Ro31-8425, Ro31-8220, and chelerythrine on antigen- or A23187-induced exocytosis. Cells were stimulated with 20 ng/ml of DNP-BSA (a) or 500 nM of A23187 (c). a and c: Time courses of antigen- or A23187-induced exocytosis without (○) or with 10 μM of Ro31-84250 (●), Ro31-8220 (▲), or chelerythrine (■). b and d: Effects of Ro31-8425, Ro31-8220, and chelerythrine on antigen- or A23187-induced exocytosis. The ratio of exocytosis in b and d was recalculated as the sum of exocytosis of each compound shown in a and c, respectively. Control in b and d means without inhibitors in a and c, respectively. The mean values of total numbers of granules in each experiment were 73–91 (a) and 76–89 (c). Values were mean ± S.E. of four separate experiments.

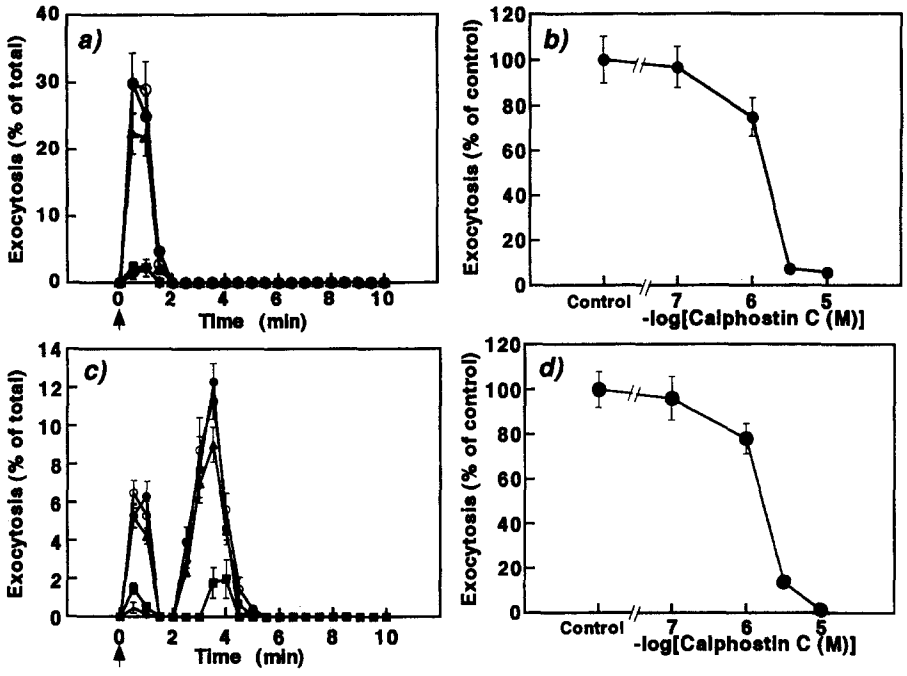

Fig. 6. Effect of calphostin C on antigen- or A23187-induced exocytosis. Cells were stimulated with 20 ng/ml of DNP-BSA (a) or 500 nM of A23187 (c). a and c: Time courses of antigen- or A23187-induced exocytosis without (○) or with 0.1 (●), 1 (▲), 3 (■), or 10 (♦) μM of calphostin C. b and d: Dose-response curves of calphostin C on antigen- or A23187-induced exocytosis. The ratio of exocytosis in b and d was recalculated as the sum of exocytosis of each dose shown in a and c, respectively. Control in b and d means without calphostin C in a and c, respectively. The mean values of total numbers of granules in each experiment were 67–82 (a) and 65–75 (c). Values were mean ± S.E. of four separate experiments.

4. Discussion

In the present study, we demonstrated that secretory processes can be visualized in RBL-2H3 cells by a video-enhanced light/fluorescence microscope. With this system, we also found that PKC and PLD were involved in antigen- and A23187-induced exocytosis not only through independent pathways but also with different time courses.

In RBL-2H3 cells, the exocytotic processes were more similar to those observed in bovine adrenal chromaffin cells, in which exocytotic events of individual granules included fusion, extrusion, swelling, omega-figure formation, and membrane retrieval [1], than those in rat peritoneal mast cells, which showed so-called compound or sequential exocytosis of granules as well as exocytosis of individual granules upon stimulation with compound 48/80 [2]. As shown in Fig. 2, upon stimulation with antigen, disappearance of the quinacrine fluorescence from a granule was observed at the same time that the exocytotic events, shown in the DIC image, were observed. As described above, quinacrine is reported to be incorporated selectively into low-pH granules, which include histamine, 5-hydroxytryptamine, and β-hexosaminidase in RBL-2H3 cells [29], these results suggest that the exocytotic events of granules, observed in this study, reflect the release of the inflammatory mediators from low-pH granules to extracellular space. Further evidence for the release of intragranular materials to extracellular space is that, although we cannot show the results here because of the limitation of the quality of photograph, release and spread of quinacrine fluorescence from granules to extracellular space was observed.

For quantitative analysis of exocytosis visualized by the video-enhanced light/fluorescence microscope, we used the ratio of the number of exocytotic granules to that of total granules, which were 0.5–0.8 μm in diameter and pre-stained with quinacrine, as the rate of exocytosis, percent of total. Although the possibility that this method may be incorrect cannot be excluded, the propriety of our estimation may be supported by the following observation The release, percent of total, calculated by the method described in the present study was similar to the results of release of histamine, 5-hydroxytryptamine, or β-hexosaminidase (data not shown, but similar results were previously reported in [18,27]).

As shown in Figs. 3–7, quantitative analysis of exocytosis directly visualized by the microscope revealed that exocytosis of granules stimulated with A23187 showed two peaks in its time course but antigen-induced exocytosis showed only one. Such characteristics, however, have not been reported before. For the quantitative analysis of exocytosis in RBL-2H3, measurements of biogenic amines or hexosaminidase release from the mass of cells have been widely used [7–23]. With measurement of hexosaminidase release from RBL-2H3 cells, A23187-induced secretion showed two phases of secretory process in its time course, but a sharp distinction between these two phases was not observed (data not shown). One explanation why two peaks of A23187-induced exocytosis were not observed with measurements of hexosaminidase or histamine release might be that the results with these methods are the averaged values of hexosaminidase or histamine release from the mass of cells but quantitation of exocytosis with the microscope shows the results in a single cell. In this paper, we observed cells only in a fixed focal plane of a cell (around 1 μm deep from the cell surface) through all experiments, but peak periods for antigen- or A23187-induced exocytosis in a different focal plane (i.e. cell surface attachment to the cover

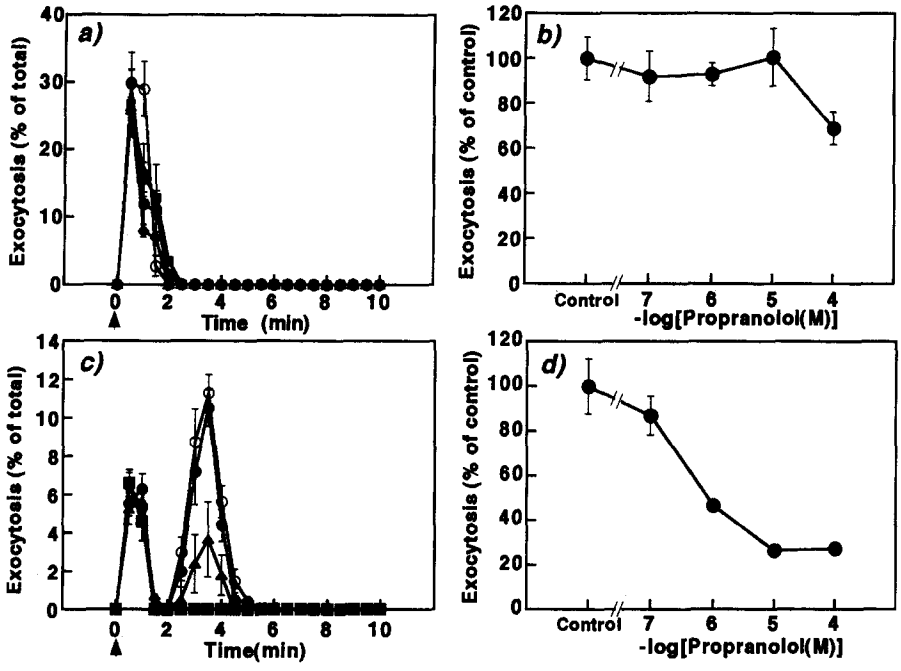

Fig. 7. Effect of propranolol on antigen- or A23187-induced exocytosis. Cells were stimulated with 20 ng/ml of DNP-BSA (a) or 500 nM of A23187 (c). a and c: Time courses of antigen- or A23187-induced exocytosis without (○) or with 0.1 (●), 1 (▲), 10 (■), or 100 (♦) μM of propranolol. b and d: Dose-response curves of propranolol on antigen- or A23187-induced exocytosis. The ratio of exocytosis in b and d was recalculated as the sum of exocytosis of each dose shown in a and c. Control in b and d means without propranolol in a and c, respectively. The mean values of total numbers of granules in each experiment were 72–89 (a) and 71–94 (c). Values were mean ± S.E. of four (experiments except 1 μM of propranolol in c) or eight (1 μM of propranolol in c) separate experiments.

glass) varied a little between each focal plane, though patterns of exocytosis were the same as the results here (data not shown). This result also supports our hypothesis on why two peaks of A23187-induced exocytosis were not observed with measurements of contents release from the mass of RBL-2H3 cells. Although the patch clamp or the voltammetric techniques have been used for the quantitative analysis of exocytosis in a single secretory cell, including mast cells, such techniques for measurement of secretion in RBL-2H3 cells have not succeeded. One advantage of quantitative analysis of exocytosis with the microscope is that we could measure exocytosis continuously not integrally but differentially in a single RBL-2H3 cell. This advantage led us to find two distinct components of A23187-induced exocytosis in RBL-2H3 cells.

In permeabilized adrenal chromaffin cells, Holz et al. [33,34] reported that there was one pathway for exocytosis that included both ATP-dependent and ATP-independent components. The ATP-independent component was a quick exocytosis, which was finished within 2 min after addition of Ca^{2+}, and the ATP-dependent component was a slow response, which was observed after 6 min of stimulation and continued up to 12 min. Also in RBL-2H3 cells, exocytosis was observed continuously up to 15 min after stimulation with antigen or A23187 [6]. The present results, however, showed that antigen- or A23187-induced exocytosis was finished within 3 min (antigen) or 7 min (A23187) after stimulation. One possible explanation is a time lag of response to the stimulation in a single cell. The fact that exocytosis of granules adhering to the glass surface was observed between 4 min and 8 min after stimulation with antigen might support this speculation. Although the possibility cannot be excluded that there might exist secretory pathways other than the exocytotic process, the present results of time course experiments reveal unique aspects of the secretory mechanisms in RBL-2H3 cells, detailed below.

Quantitative analysis of exocytosis revealed several new aspects for the mechanisms of exocytosis, such that A23187-induced exocytosis is mediated through activation of both PKC and PLD and that antigen-induced exocytosis is caused mainly by activation of PKC. We have already reported that by using washed, streptolysin *O*-permeabilized RBL-2H3 cells the elevation of $[Ca^{2+}]_i$ and provision of the Ca^{2+}-dependent β or the Ca^{2+}-independent δ isozyme of PKC completely restored the secretory response to antigen [18], suggesting that PKC is necessary for exocytosis. And also, A23187 induced a rise in concentration of cytosolic Ca^{2+}, the activation of PLD, and redistribution of PKC, followed by secretion of histamine (H.M.S. Gonzaga, K. Ozawa, K. Yamada, and M.A. Beaven, manuscript in preparation). Further implications of mechanisms for exocytosis, however, have not been clarified, because of the limitation of the previous methodology, for example, limitation of time course experiments, which cannot clearly identify the difference underlying the activation of PLD and PKC. As shown in Figs. 4, 5 and 7, quantitative analysis of A23187-induced exocytosis clearly indicated that activation of both PKC and PLD promotes exocytosis through different pathways also with different time courses. Time courses for the activation of PKC [18,19] or PLD (H.M.S. Gonzaga, K. Ozawa, K. Yamada, and M.A. Beaven, manuscript in preparation) were parallel to those for PKC- or PLD-induced exocytosis shown in Figs. 3–7. In contrast, it is also suggested that the activation of PKC is involved in antigen-induced exocytosis but the activation of PLD is not involved in it

(see Figs. 4, 5 and 7). Although Nakashima et al. [35] reported that stimulation of RBL-2H3 cells with antigen caused production of 1,2-diacylglycerol through the activation of PLD, independent of activation of PKC and elevation of Ca^{2+}, the present results suggest that activation of PLD pathways is not involved in antigen-induced secretion of granules or its contribution to the secretion is not so high at least up to 10 min after the stimulation with antigen.

In RBL-2H3 cells, A23187 induced a rise in concentration of cytosolic Ca^{2+}, activation of PLD, and redistribution of PKC, followed by secretion of histamine (H.M.S. Gonzaga, K. Ozawa, K. Yamada, and M.A. Beaven, manuscript in preparation). Propranolol inhibited A23187-induced diacylglycerol accumulation, through the activation of PLD, and secretion of hexosaminidase (H.M.S. Gonzaga, K. Ozawa, K. Yamada, and M.A. Beaven, manuscript in preparation) in RBL-2H3 cells. Although the question remains whether activation of PLD is involved in A23187-induced secretion, the results with propranolol, shown in Fig. 7c, strongly indicate the possible involvement of activation of PLD pathways in the second peak of A23187-induced exocytosis. Results using Ro31-8220, reported to inhibit PKC but not PLD [36,37], and chelerythrine as well as Ro31-8425 (Fig. 5) also support the involvement of activation of PKC only in the first peak of A23187-induced exocytosis and PLD in the second peak. Recently our group demonstrated using wortmannin that PI3-kinase and MLCK play essential roles in antigen-induced exocytosis and the activation of MLCK is involved in the first peak of A23187-induced exocytosis in RBL-2H3 cells [29]. With permeabilized RBL-2H3 cells, it was also suggested that PI3-kinase acts upstream of PKC, which transduces stimulatory signals for release process in RBL-2H3 cells [13], and MLCK downstream of PKC (K. Ozawa, Y. Nonomura and T. Masujima, manuscript in preparation). Kitani et al. [31] reported that PKC-inhibitors or wortmannin (at the concentration as a MLCK inhibitor) showed only 50% inhibition of A23187-induced histamine release from RBL-2H3 cells. This result is related to the present work.

Acknowledgements: We thank Dr. Susumu Terakawa (Hamamatsu University School of Medicine, Japan) for the excellent discussion on the construction of a video-enhanced light/fluorescence microscope. This work was carried out with financial support from the Japan Society for the Promotion of Science and the Human Science Foundation and a Grant-in-Aid for Developmental Scientific Research from the Ministry of Education, Science and Culture, Japan. This work was partly supported by the Fugaku Trust for Medical Research.

References

[1] Terakawa, S., Fan, J.-H., Kumakura, K. and Ohara-Imaizumi, M. (1991) Neurosci. Lett. 123, 82–86.
[2] Kawasaki, Y., Saitoh, T., Okabe, T., Kumakura, K. and Ohara-Imaizumi, M. (1991) Biochem. Biophys. Acta 1067, 71–80.
[3] Terakawa, S. and Suzuki, Y. (1991) Biochem. Biophys. Res. Commun. 176, 466–472.
[4] Segawa, A., Terakawa, S., Yamashina, S. and Hopkins, C.R. (1991) Eur. J. Cell. Biol. 54, 322–330.
[5] Seldin, D.C., Adelman, S., Austen, K.F., Stevens, R.L., Hein, Caulfield, A., J.P. and Woodbury, R.G. (1985) Proc. Natl. Acad. Sci. USA 82, 3871–3875.
[6] Beaven, M.A. and Cunha-Melo, J.R. (1988) Prog. Allergy 42, 123–184.
[7] Metzger, H. (1992) J. Immunol. 149, 1477–1487.
[8] Jouvin, M.-H.E., Adamczewski, M., Numerof, R., Letourneur, O., Valle, A. and Kinet, J.-P. (1994) J. Biol. Chem. 269, 5918–5925.
[9] Benhamou, M., Gutkind, J.S., Robbins, K.C. and Siraganian, R.P. (1990) Proc. Natl. Acad. Sci. USA 87, 5327–5330.
[10] Benhamou, M. and Siraganian, R.P. (1992) Immunol. Today 13, 195–197.
[11] Benhamou, M., Stephan, V., Robbins, K.C. and Siraganian, R.P. (1992) J. Biol. Chem. 267, 7310–7314.
[12] Park, D.J., Min, H.K. and Rhee, S.G. (1991) J. Biol. Chem. 266, 24237–24240.
[13] Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y. and Matsuda, Y. (1993) J. Biol. Chem. 268, 25846–25856.
[14] Grushalla, R.S., Dinh, T.T. and Kennerly, D.A. (1990) J. Immunol. 144, 2334–2342.
[15] Lin, P., Wiggan, G.A. and Gilfillan, A.M. (1991) J. Immunol. 146, 1609–1616.
[16] Jones, S.V.P., Choi, O.H. and Beaven, M.A. (1991) FEBS Lett. 289, 47–50.
[17] Millard, P.J., Ryan, T.A., Webb, W.W. and Fewtrell, C. (1989) J. Biol. Chem. 264, 19730–19739.
[18] Ozawa, K., Szallasi, Z., Kazanietz, M.G., Blumberg, P.M., Mischak, H., Mushinski, J.F. and Beaven, M.A. (1993) J. Biol. Chem. 268, 1749–1756.
[19] Ozawa, K., Yamada, K., Kazanietz, M.G., Blumberg, P.M. and Beaven, M.A. (1993) J. Biol. Chem. 268, 2280–2283.
[20] Peleg, I., Ludowyke, R.I., Beaven, M.A. and Adelstein, R.S. (1992) J. Lab. Clin. Med. 120, 675–680.
[21] Teshima, R., Suzuki, K., Ikebuchi, H. and Terao, T. (1989) Mol. Immunol. 26, 641–648.
[22] Fukamachi, H., Takei, M. and Kawakami, T. (1993) Int. Arch. Allergy Immunol. 102, 15–25.
[23] Santini, F. and Beaven, M.A. (1993) J. Biol. Chem. 268, 22716–22722.
[24] Monck, J.R. and Fernandez, J. M (1992) J. Cell. Biol. 119, 1395–1404.
[25] Jonathan, R. and Fernandez, J.M. (1994) Neuron 12, 707–716.
[26] Alvarez de Toledo, G., Fernandez-Chacon, R. and Fernandez, J.M. (1993) Nature 363, 554–558.
[27] Cunha-Melo, J.R., Gonzaga, H.M.S., Ali, H., Huang, F.L., Huang, K.-P. and Beaven, M.A. (1989) J. Immunol. 143, 2617–2625.
[28] Ozawa, K., Masujima, T., Ikeda, K., Kodama, Y. and Nonomura, Y. (1996) Biochem. Biophys. Res. Commun. 222, 243–248.
[29] Kolber, M.A. and Henkart, P.A. (1988) Biochim. Biophys. Acta 939, 459–466.
[30] Kitani, S., Teshima, R., Morita, Y., Ito, K., Matsuda, Y. and Nonomura, Y. (1992) Biochem. Biophys. Res. Commun. 183, 48–54.
[31] Bruns, R.F., Miller, F.D., Merriman, R.L., Howbert, J.J., Heath, W.F., Kobayashi, E., Takahashi, I., Tamaoki, T. and Nakano, H. (1991) Biochem. Biophys. Res. Commun. 176, 288–293.
[32] Shariff, A. and Luna, E.J. (1992) Science 256, 245–247.
[33] Holz, R.W., Bittner, M.A., Peppers, S.C., Senter, R.A. and Eberhard, D.A. (1989) J. Biol. Chem. 264, 5412–5419.
[34] Bittner, M.A. and Holz, R.W. (1992) J. Biol. Chem. 267, 16219–16225.
[35] Nakashima, S., Fujimiya, H., Miyata, H. and Nozawa, Y. (1991) Biochem. Biophys. Res. Commun. 177, 336–342.
[36] Uings, I.J., Thompson, N.T., Randall, R.W., Spacey, G.D., Bonser, R.W., Hudson, A.T. and Garland, L.G. (1992) Biochem. J. 281, 597–600.
[37] Wilkinson, S. and Hallam, T.J. (1994) Trends Pharmacol. Sci. 15, 53–57.